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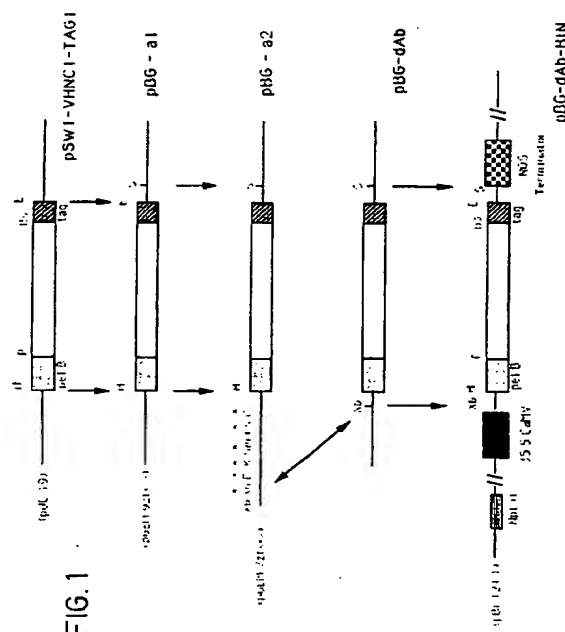
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Plasmid vectors for gene expression in plants.

A plasmid vector for cloning and expressing sequences in plants is disclosed, said vector being particularly suitable for cloning immunoglobulin chains or portions of the same to be employed for introducing molecules capable of conferring new characteristics to the plant so transformed, such as resistance to chemical compounds or pathogenous agents.



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This invention relates to plasmid vectors for gene expression in plants.

More particularly, this invention relates to plasmid vectors that allow exogenous sequences to be expressed in plants, such sequences coding preferably for variable regions of immunoglobulins, such vectors being intended for giving the transformed plant some specific characteristics, and in addition the invention also relates to the process of transforming plants by means of said vectors as well as to the employment of said vectors for giving the transformed plants some new characters.

The numbered list of bibliographic references is reported at the end of this disclosure and the documents are cited with the numerical reference in parentheses ( ).

The employment of bacteria belonging to the genus *Agrobacterium* for transferring exogenous genes into the nuclear genome of plant cells is already known from the prior art (1, 2).

Among the genes or portions of the same which are potentially usable for being introduced into plant cells there are those coding for the chains of immunoglobulins, because the virtually unlimited inventory of this family of molecules can be widely employed both as a source of specific reagents for biochemical and physiological studies in which it is desired to modulate or to inhibit the action of some definite gene products, and for introducing exogenous molecules in a stable way that are capable of conferring new characters to the receptor plant, as for instance the resistance to chemical products or to pathogenous agents. This is possible because it has been shown that an efficient assembling of antibody chains is obtainable for forming the active molecule, even in non lymphoid cells (3, 4).

Employing the transformation technique which is mediated by *Agrobacterium*, the production of complete antibody molecules has been obtained in transgenic plants by crossbreeding two plants already transformed with recombinant vectors that expressed respectively gamma- or kappa-immunoglobulin chains (5, 6) or, with one single step, employing as an expression vector a vector comprising two promoters, downstream of which some sequences coding for two different immunoglobulin chains had been sub-cloned (7). Such vectors, and the processes realizable with the same, do not appear to be easily employable, as in the first instance they require long and difficult crossbreeding experiments and in the second instance the vector is not adaptable to the cloning and the expression of immunoglobulins different from those that have been already.

Simpler forms of antibody molecules, recently identified are certainly more suitable for their functional expression in plants, eluding the mentioned assembling difficulties of immunoglobulin chains and of correct transportation of synthesized molecules into

the cell compartment wherein the target molecule is present. As is well known, the antibody portion which is capable of reacting with an antigen is represented by the variable domains of the heavy chains (VH) and of the light chains (VL) of immunoglobulins (8). Recently, the cloning and expression of variable domains of the transposed heavy chain (VH) in *E. coli* has been reported (9), and it has been shown that the variable domain VH alone, without the contribution of the domain VL, is often capable of binding the antigen with a high value of the affinity constant (Patent Application EP 0368684). Such domains, which are called single-domain antibodies (dAbs), represent molecules which are extremely versatile, with respect to the whole antibodies, in experiments of transfer and gene expression.

At last, a chimeric gene has been constructed, comprising sequences coding both for VH chain and for VL chain of a specific antibody; said sequences are conveniently separated by a peptide leading to a correct tridimensional structure of the antibody chains, even they are comprised into a linear polypeptidic chain (sc Fv, 10).

Accordingly, there is clearly the need for a plasmid vector for cloning and expressing exogenous genes or portions of them in plants, which vector can be easily and generally employed for genes coding for immunoglobulin chains or portions of immunoglobulin chains, and even for dAbs or scFv.

The authors of the present invention have built a plasmid vector satisfying such requirements, as it is easily employable for cloning any exogenous gene, or a portion of the same, said vector, when introduced into plants, allowing a stable and efficient transcription to be realized. The vector is particularly suitable for introducing genes which code for immunoglobulin chains or for portions of the same, which genes are obtained by the amplification of genes for hybridoma line immunoglobulins, as well as for dAbs or scFv.

Accordingly, it is the object of this invention a plasmid vector for cloning and expressing exogenous genes in plants, said vector comprising at least:

- a sequence coding for a selectable marker in plants;
- a sequence capable of promoting the efficient and correct transcription (the promoter sequence) of said exogenous gene in plants;
- restriction sites for the unidirectional insertion of said exogenous gene;
- a sequence detecting the correct and efficient transcription of said promoter at the 3' site of said restriction sites;
- a sequence capable of promoting the efficient and correct termination of transcription (the terminator sequence) of said exogenous gene in plants.

Again according to this invention, said marker sequence codes for a protein having a phosphotransfer-

ase II neomycin activity (NPTII), conferring resistance to plants grown on culture media containing kanamycin; said promoter comprises the elements that regulate the gene 35S of the CaMV virus (the virus of the cauliflower mosaic); said restriction sites comprise the site Pst I at 5' position and the site BstE II at the 3' position; said detector sequence codes for a peptide which is capable of reacting with a known antibody; wherein said peptide is the TAG peptide and said antibody is an anti-TAG antibody; said terminator is the gene terminator of the nopal synthetase.

Further according to this invention said exogenous gene codes for immunoglobulin chains, preferably comprised in the group of the immunoglobulin G (IgG) chains, and even more preferably in the group of the heavy chains (VH) and of the light chains (VL) or portions of the same. Alternatively, said portions comprise single antibody domains (dAbs).

Alternatively said portions comprise single chain antibodies (scFv).

Again according to this invention, said immunoglobulin chains are produced by hybridoma cell lines; according to a particular embodiment, said hybridoma produces the monoclonal antibody NCI/34HL.

According to a preferred embodiment of this invention, said vector also comprises a sequence coding for a signal peptide, which covalently linked to the NH2 terminal of the exogenous protein, capable of promoting the transportation and the extracellular secretion of said exogenous protein.

A preferred embodiment of this invention consists in the plasmid pBG-dAb-BIN, which is disclosed in Figure 1 and has been deposited, after transforming E.coli with the same, with the NCAIM in agreement with the Budapest Treaty, under the accession number 001144 on 31st May, 1991.

Again, it is an object of this invention a process for cloning said coding sequences in the vector disclosed and for transforming plants by means of said chimeric vector, capable of promoting the correct and efficient transcription of said sequences in transformed plants.

Said process comprises the following steps:

- inserting the DNA portion coding for said coding sequence into said vector by means of restriction sites so as to obtain a chimeric vector;
- transforming bacteria belonging to the genus *Agrobacterium* by means of said chimeric vector;
- infecting the plant to be transformed with said transformed bacteria;
- selecting obtained transgenic plants with said marker that is expressed in plants;
- checking the expression levels of said coding sequence in transgenic plants by means of the product coded by said detector sequence.

According to a particular embodiment of this invention, said immunoglobulin chains react in a specific way with chemical compounds or pathogenous agents, giving to the transformed plant resistance to

said chemical compounds or to said pathogenous agents.

Also plants transformed by means of said vectors, or parts of said plants, including the reproduction material thereof, are an object of this invention.

This invention will be disclosed in the following with reference to some application examples of the same which are not limitative of the invention itself, with reference to the following figures wherein:

- Figure 1 represents a scheme illustrating a way to build one of the vectors which are the object of this invention; the restriction sites labelled by an asterisk (\*) have been deleted. Bs: BstE II, C: Cla I, Cs: Csp45 I, E: Eco RI, H: Hind III, K: Kpn I, P: Pst I, S: Sac I, Sm: Sma I, Xb: Xba I, Xh: Xho I;
- Figure 2 represents the map of the region that characterizes one of the vectors which are the object of the present invention.

In the examples disclosed in the following, the techniques which are well known to those who are skilled in the art are carried out according to reference (17).

#### Example 1

##### Constructing the vector pBG-dAb-BIN

The vector pSW1-VHNCI-TAG1 described by (9) has been employed as the starting vector just for exemplification purposes, but it is to be understood that alternative construction ways are equally possible.

With reference to Figure 1, the fragment Hind III-Eco RI has been excised and subcloned into the sites Hind III-Eco RI of the vector pGEM 7zf(+) (Promega) in which the "polylinker" sites labelled with an asterisk had been removed by means of digestion and treatment with Klenow's fragment of the DNA-polymerase. Following the digestion with Xba I and Sac I, it was possible to subclone the fragment into the vector pB1121 (Clontech Laboratories Inc., Ca).

The removal of the polylinker restriction sites causes the plant active 35S CaMV promoter to be suitably close to the sequence to be transcribed, conferring an optimal *in vivo* transcription efficiency to the system. By means of the sites Pst I and BstE II, it is possible to insert any exogenous gene into the vector. In particular, as these sites are contained within the primers employed for amplifying from hybridoma lines the sequences that code for immunoglobulin chains (11), such sequences can be easily introduced in said sites without additional steps. The vector so obtained has been called pBG-dAb-BIN and it has been deposited after transforming E.coli with the same and in agreement with the Budapest Treaty with the NCAIM under the accession number 001144, on 31st May, 1991.

## Example 2

### Constructing the vector pBG-NC1-BIN and transforming *N.benthamiana* with the same

A DNA fragment coding for the heavy chain of the monoclonal antibody NC1/34 HL disclosed in (12) has been introduced into the vector pBG-dAb-BIN just for exemplification purposes, said antibody being capable of recognizing a neuropeptide, i.e. the tachyquinine or substance P, employing the sites PstI and BstE II, so originating the plasmid pBG-NCI-BIN.

Plants of the species *N. benthamiana* have transformed by means of the plasmid obtained employing techniques which are already known to those who are skilled in the art. Stated briefly, the plasmid pBG-NC1-BIN has been introduced into the strain LBA4404 of *A.tumefaciens* (13) as disclosed in reference (14) and disks obtained from the leaves of *N.benthamiana* plants, 4 weeks of age, were infected with the bacteria transformed as disclosed in reference (15). After 3-4 weeks, small plants were evidenced that regenerated on a culture medium suitable to the formation of sprouts (MS, containing 1 mg/ml of 3-indolbutyric acid (IBA), 1 mg/ml of 6benzylaminopurine (BAP) and 30 g/l of sucrose), containing 100 ug/ml of Kanamycin with a 95 % frequency.

Each leaf explantation gave origin to  $13.8 \pm 1.8$  sprouts. The regenerated sprouts were caused to root on a selective medium with 25 ug/ml of Kanamycin containing 1/2 MS with 0.05 mg/ml of IBA and 30 g/ml of sucrose. The plants so obtained showed resistance to Kanamycin through NPTII activity as disclosed in (16). More than 65 % of the plants turned out to be positive.

The putative transgenic plants multiplied *in vitro* showed normal morphology with respect to the control plants. DNA was extracted from independent plants and hybridized through "Southern" technique employing as probe the fragment Hind III-Eco RI of the pBG-dAb plasmid, putting into evidence a number of 1-5 copies of transformant gene per plant.

## Example 3

### Expression of the NC1/34 antibody in plants: analysis of mRNA and proteins

Cytoplasmic RNA was extracted from the leaves of 15 independent NPTII-positive transformants at the same physiological stage and it was hybridizes through the "Northern" technique employing the same probe as that employed in example 2. Five plants out of those analysed turned out to be positive, with differences in the expression level not ascribable to the number of copies of the gene or to mRNA degradation phenomena but, quite likely, ascribable to position effects in the plant genome.

Soluble protein extracts were prepared from the leaves of transgenic plants and from the leaves of control plants, and the expression of the protein NC1/34 was tested by means of the "Western" technique, with the monoclonal antibody 9E10 that recognizes the peptide TAG at the C-terminal of the recombinant protein (18). All extracts out of three extracts from transgenic plants showed a protein band which was absent from the control plants. Moreover, the expression levels of the transgenic protein were well correlated with the specific RNA levels in the same plants. The relative amount of the expressed protein varies in the range between 0.1 % and 1 % of the total soluble proteins.

## Example 4

### Locating the protein

The distribution throughout the tissues was investigated by immunofluorescence in sections obtained from leaves, flowers and stems of three transgenic plants, and a strong positive signal was detected in particular in the leaves, the meristems and the flowers.

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## Claims

1. A plasmid vector for cloning and expressing exogenous genes in plants, said vector comprising at least
  - a sequence coding for a selectable marker in plants;
  - a sequence capable of promoting the efficient and correct transcription (the promoter sequence) of said exogenous genes in plants;
  - restriction sites for the unidirectional insertion of said exogenous gene;
  - a sequence capable of detecting the correct and efficient transcription of said promoter at the 3' of said restriction sites;
  - a sequence capable of promoting the efficient and correct termination of transcription (the terminator sequence) of said exogenous

gene in plants.

2. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 1, wherein said marker sequence codes for a protein having a phosphotransferase II neomycin activity (NPTII) that confers resistance to plants grown in a culture medium containing Kanamycin.
3. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said promoter comprises the regulative elements of the 35S CaMV virus gene (the cauliflower mosaic virus).
4. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said restriction sites comprise the site Pst I at the 5' position and the site BstE II at the 3' position.
5. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said detecting sequence codes for a peptide which is capable of reacting with a known antibody.
6. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 5 wherein said peptide is the TAG peptide and said antibody is an anti-TAG antibody.
7. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said terminator is the terminator of the nopal synthetase gene.
8. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said exogenous genes code for immunoglobulin chains.
9. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 8, wherein said immunoglobulin chains are included in the group of the chains of immunoglobulins G (IgG).
10. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 9, wherein said IgG chains are included in the group of the heavy chains (VH) and of the light chains (VL) or of portions of the same.
11. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 10 wherein said portions comprise single antibody

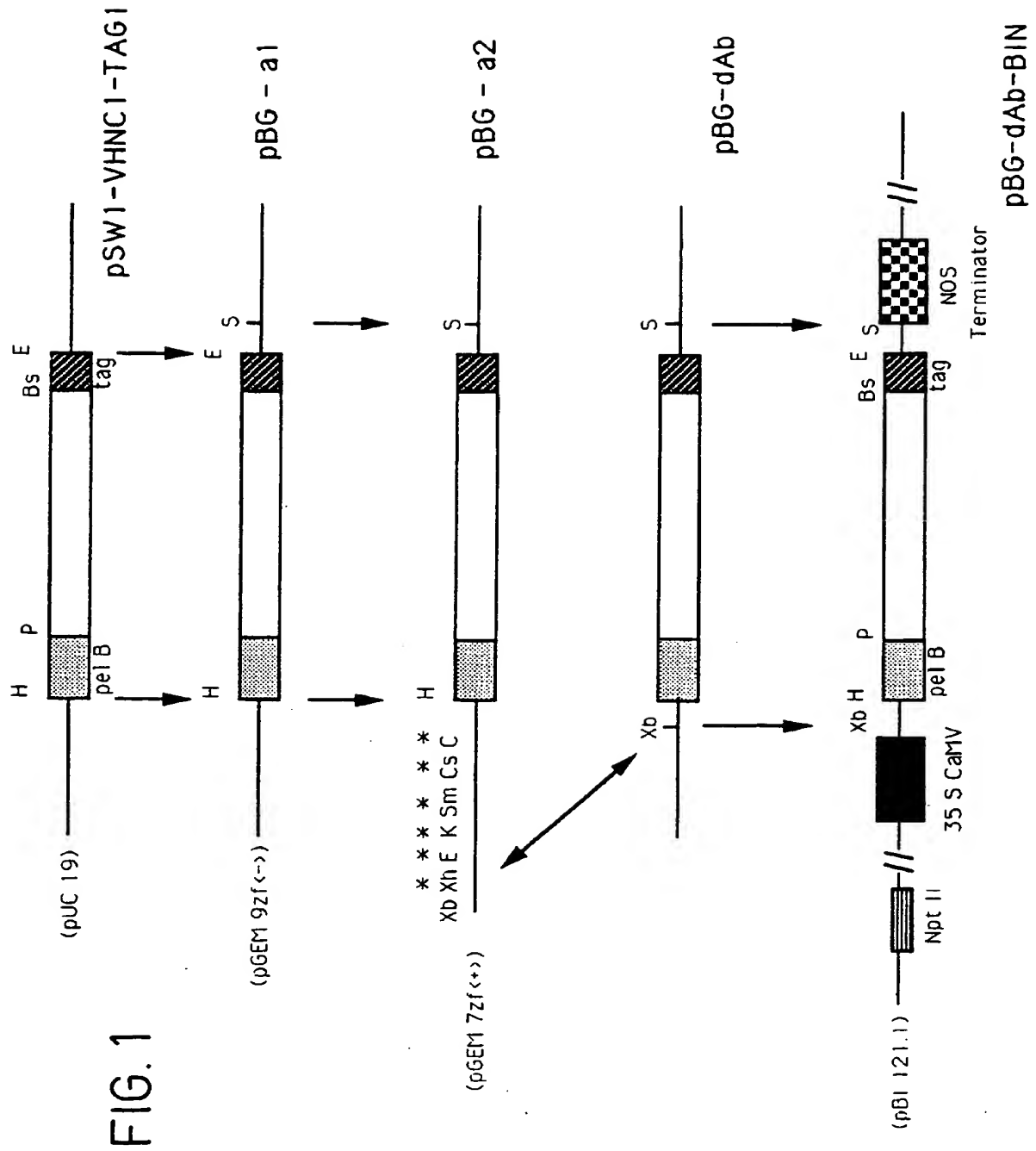
domains (dAbs).

12. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 10 wherein said portions comprise single chain antibodies (scFv). 5
13. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims 8-12 wherein said immunoglobulin chains are produced by hybridoma cell lines. 10
14. A plasmid vector for cloning and expressing exogenous genes in plants according to claim 13, wherein said hybridoma cell line produces the monoclonal antibody NC1/34HL. 15
15. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims 8-13, wherein said immunoglobulin chains react in a specific way with chemical compound or pathogenous agents, conferring to the transformed plant a resistance to said chemical compound or said pathogenous agents. 20 25
16. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims wherein said vector comprises a sequence coding for a signal peptide, which is covalently linked to the NH2 terminal of the exogenous protein, capable of promoting the transportation and extracellular secretion of said exogenous protein. 30 35
17. A plasmid vector for cloning and expressing exogenous genes in plants according to any one of the preceding claims, which consists of the plasmid pBG-d-Ab-BIN, which has been deposited, after transforming E.coli with the same, with the NCAIM under the accession number 001144 on 31st May, 1991, in agreement with the Budapest Treaty. 40
18. A cloning process in the vector according to any one of the preceding claims, and a plant transformation process by means of said chimeric vector which is capable of promoting the correct and efficient transcription of said cloned sequences in the transformed plants. 45 50
19. A cloning and plant transformation process according to claim 14, said process comprising the following steps: 55
  - the insertion of a coding sequence into said vector by means of restriction sites;
  - the transformation of bacteria belonging to the genus Agrobacterium by means of said

chimeric vector;

- infection of the plant to be transformed with said transformed bacteria;
- selection of the transgenic plants obtained by means of said marker that is expressed in plants;
- check of the expression levels of said coding sequence in transgenic plants by means of the product coded by said detector sequence.

20. Plants or parts of the same transformed by means of vectors according to any one of the preceding claims 1-17.
21. Parts of a plant according to claim 20, wherein said parts comprise the reproduction material.
22. Plants or parts thereof according to the preceding claims 20 or 21, said plants or parts being obtained by means of the process according to the preceding claims 18 or 19.



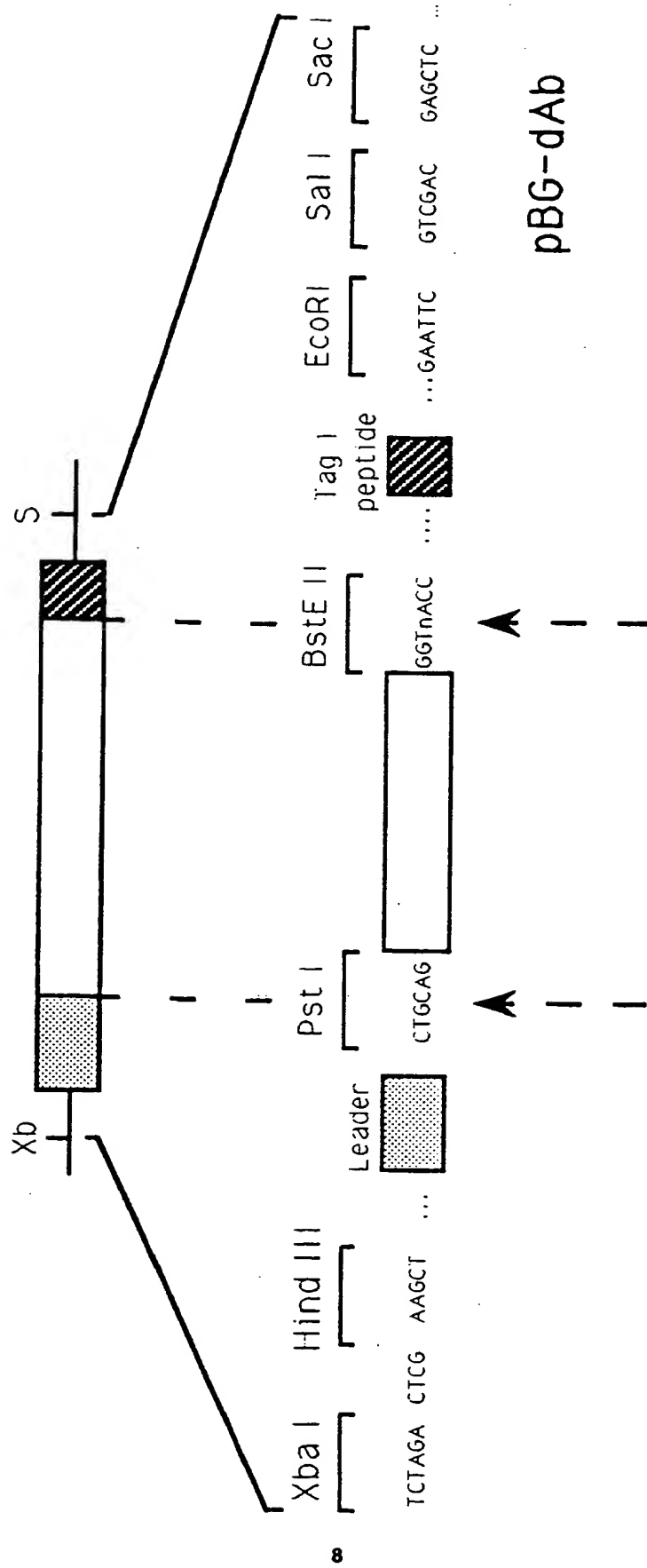


FIG. 2